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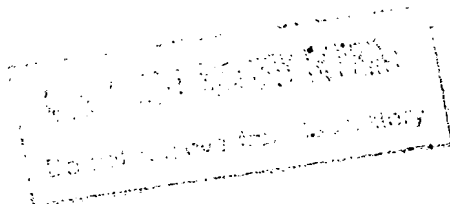
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Applicant: **IMPERIAL CHEMICAL INDUSTRIES PLC**  
**Imperial Chemical House Millbank**  
**London SW1P 3JF (GB)**

(72)

Inventor: **Bridges, Ian George**  
**Box 30A RRI**  
**Slater Iowa 50244 (US)**

**Schuch, Wolfgang Walter**  
**14 Greenfinch Close Heathlake**  
**Park Crowthorne (GB)**

**Grierson, Donald**  
**6 Tyler Court**  
**Shepshed Leicestershire (GB)**

(74)

Representative: **Roberts, Timothy Wace et al**  
**Imperial Chemical Industries PLC Legal Department :**  
**Patents PO Box 6 Bessemer Road**  
**Welwyn Garden City Herts, AL7 1HD (GB)**

(54) **Tomatoes.**

(57) Fruit, especially tomatoes, having lowered expression of fruit-softening enzymes caused by antisense gene expression, and seeds therefrom.

*fruit / polygalacturonase / enzyme / tomato*

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DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
X	EP-A-0 240 208 (CALGENE) * Page 4, lines 29-30; page 5, lines 16-19; page 11, lines 20-50; claims * ---	1-6	A 01 H 5/08 A 01 H 5/10 A 01 H 1/00 // C 12 N 15/00
O,X	JOURNAL OF CELLULAR BIOCHEMISTRY, supplement 12C, 1988, ABSTRACTS OF THE 17TH ANNUAL MEETINGS, UCLA SYMPOSIA ON MOLECULAR & CELLULAR BIOLOGY, 28th February-10th April 1988, pages 126,147, abstract no. L 041, Alan R. Liss, Inc., New York, US; D. GRIERSON et al.: "Physiological and genetic factors governing the expression of the polygalacturonase gene in ripening tomatoes" * Abstract no. L 041 * ---	1-6	
O,X	JOURNAL OF CELLULAR BIOCHEMISTRY, supplement 12C, 1988, ABSTRACTS OF THE 17TH ANNUAL MEETINGS, UCLA SYMPOSIA ON MOLECULAR & CELLULAR BIOLOGY, 28th February-10th April 1988, pages 126,148, abstract no. L 043, Alan R. Liss, Inc., New York, US; W.R. HIATT et al.: "Expression of selected genes during tomato fruit maturation and ripening" * Abstract L 043 * ---	1-6	
P,X	EP-A-0 271 988 (ICI) * Whole document * ---	1-7	
P,X	WO-A-8 809 334 (CALGENE) * Whole document * -----	1-6	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 14-11-1989	Examiner MADDOX A.D.
<b>CATEGORY OF CITED DOCUMENTS</b> X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			



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PLC  
Imperial Chemical House Millbank  
London SW1P 3JF(GB)

72 Inventor: Bridges, Ian George  
Box 30A RRI  
Slater Iowa 50244(US)  
Inventor: Schuch, Wolfgang Walter  
14 Greenfinch Close Heathlake  
Park Crowthorne(GB)  
Inventor: Grierson, Donald  
6 Tyler Court  
Shepshed Leicestershire(GB)

74 Representative: Roberts, Timothy Wace et al  
Imperial Chemical Industries PLC Legal  
Department : Patents PO Box 6 Bessemer  
Road  
Welwyn Garden City Herts, AL7 1HD(GB)

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## TOMATOES

This invention relates to novel fruit and seeds, and more specifically but not exclusively to novel tomato fruit and seeds produced by genetic modification of the natural tomato plant.

In our co-pending European patent specification No 87309853.7 (now published as 271988) we disclose plants, in particular tomato plants, that have been genetically modified by inclusion of DNA constructs capable of expressing RNA complementary to RNA encoding fruit-softening enzymes. We have now shown that such tomato plants are able to produce fruit and fertile seed. Furthermore fruit of the selfed progeny of such plants show surprising properties, which may include an unexpectedly high level of inhibition of such fruit-softening enzymes.

According to the present invention we provide fruit, especially tomato fruit, having a reduced level of expression of one or more fruit-ripening enzymes, resulting from the presence in the plant genome of DNA capable of generating antisense RNA complementary to the RNA that generates such fruit-ripening enzymes. Preferably such enzymes are fruit-softening enzymes, eg cell wall softening enzymes. We further provide fertile seed, derived from such fruit, which may be cultured to produce similar fruit.

Fruit according to our invention may be obtained by growing and cropping, using conventional methods, the tomato plants described in our European Patent Application No 87309853.7.

Methods for making such plants, by genetic manipulation of known tomato plants, are fully described in the aforesaid European Patent Application No 271988 and in US Application Serial 119614, the complete disclosures of which applications are incorporated herein by reference.

A convenient method of obtaining such plants is to grow the seeds from tomato fruit according to the invention. We have deposited samples of such seeds with the National Collections of Industrial and Marine Bacteria (hereinafter NCIMB), Torry Research Station, PO Box 31, 135 Abbey Road, Aberdeen AB9 8DG, Scotland, under the reference numbers 40015 and 40134.

Fruit ripening is a complex physiological and biochemical process. It requires the activation of specific genes which are required to give fruit the desirable characteristics important for human consumption, and for industrial processing for consumption. In tomatoes, the end product of the mature fruit is characterised by good, intense and even colour production, specific flavour components and texture suitable for the intended end use (consumption fresh or industrial processing). In bio-

chemical terms these attributes are brought about through the action of enzymes that are responsible for the breakdown of chlorophyll and the synthesis of lycopene  $\beta$ -carotene for colour production, the synthesis of flavour components through lipoxygenase, the synthesis of enzymes involved in the maintenance of water and mineral balance, enzymes, controlling vitamin production, and most importantly for the purposes of the present invention, the production of cell wall degrading enzymes leading to fruit softening.

Preferred fruit according to our invention show reduced levels of expression of the polygalacturonase or the pectin methylesterase gene, or both. This may result from the presence in the genome of one, or several, antisense constructs to either or both genes.

Both pectinesterase and polygalacturonase are involved in the biochemical conversion of pectic cell wall substances during fruit ripening. Pectins demethylated by pectinesterase are believed to be the substrate for polygalacturonase. According to one hypothesis, polygalacturonase solubilises pectic fragments during early ripening stages, and reduces the molecular weight of pectic fragments during later stages of ripening. In fruit according to the invention, the reduction in molecular weight of solubilised pectin is inhibited.

Other enzymes that may be involved in cell wall metabolism, especially cell wall breakdown in ripening fruit, including the following:

xylanases;  $\alpha$ -galactosidase;  $\beta$ -galactosidase; cellulase ( $\beta$ -1,4-glucanase); peroxidase; hemicellulase; isoperoxidase; glucosidases; mannosidases; arabinosidases;  $\beta$ -fructofuranosidases; trehalase;  $\beta$ -xylosidase; acid phosphatase; rhaminase;  $\beta$ -1,3-glucanase; enzymes of calcium metabolism; arabinofuranosidase.

As well as tomatoes, these enzymes, or some of them, may be active in fruit ripening of other fruit besides tomatoes, for example strawberries, melons, cantaloups, peppers and avocados. Accordingly, our invention includes such fruit having a reduced level of expression of any of the aforesaid enzymes.

Preliminary results indicate that tomatoes of the invention having a reduced level of expression of polygalacturonase retain their firmness for a longer period after harvesting than similar tomatoes having normal levels of expression of fruit softening enzymes. They are consequently expected to soften more slowly on the plant, be harder at the time of harvesting and have a longer shelf life, with potentially increased resistance to infection. It is useful to be able to harvest fruit later when flavour,

aroma and colour may have developed more fully. Fruit according to our invention may also show increased solids content and altered pectin and cell wall components, with consequent processing advantages. These advantages include energy savings during tomato puree production, increased insoluble solids, and improved gelling qualities and colour of puree.

Seeds according to our invention may be obtained from fruit according to our invention by conventional methods. For example, tomato seeds are separated from the pulp of the ripe fruit and dried, following which they may be stored for one or more seasons.

The fruit of our invention may be sold for immediate consumption, raw or cooked, or processed by canning or conversion to soup, sauce or paste. Equally, they may be used to provide seeds according to the invention.

The tomato plants described in European Patent Application No 87309853.7 are heterozygous for the antisense constructs. The seeds obtained from self fertilisation of such plants are a population in which the antisense constructs behave like single Mendelian genes and are distributed according to Mendelian principles: eg, which such a plant contains only one copy of the construct, 25% of the seeds contain two copies of the construct, 50 % contain one copy and 25% contain no copy at all. Thus not all the offspring of selfed tomato plants according to European Patent Application No 87309853.7 produce fruit and seeds according to the present invention, and those which do may themselves be either heterozygous or homozygous for the defining trait. Some homozygous fruit according to the invention (eg those obtained from NCIB 40134) are found to show extremely low levels of expression of the fruit-ripening enzyme, eg 5% or less as compared with similar known fruit.

It is convenient to maintain a stock of seed which is homozygous for the antisense construct. All crosses of such seed stock will contain at least one copy of the construct, and self-fertilized progeny will contain two copies, i.e. be homozygous in respect of the character. Such homozygous seed stock may be conventionally used as one parent in F1 crosses to produce heterozygous seed for marketing. Such seed, and fruit derived from it, form further aspects of our invention. It is possible according to our invention to transform two or more plants with different antisense constructs and to cross the progeny of the resulting lines, so as to obtain seed of plants which contain two or more antisense constructs leading to reduced expression of two or more fruit-ripening enzymes.

The following examples illustrate our invention.

## EXAMPLE 1

Example 40 of our European Patent Application No 87309853.7 describes the preparation of a tomato plant containing the PG antisense vector pJR16A incorporated in its genome. This plant (referenced as GR16, and derived from Lycopersicon esculentum Mill cv Ailsa Craig) was grown in a growth room under standard conditions suitable for growing tomatoes. The flowers are self-fertilised, and 15 resulting fruit collected. Analysis of these by the method of G A Tucker, N G Robertson, D Grierson, (European Journal of Biochemistry, 112 p 119-124, 1980) indicated a low level of expression of the polygalacturonase enzyme, approximately 10% of that in corresponding conventional tomato fruit at similar stage of development. Fruit were also tested for firmness, and showed increased firmness throughout ripening. Seeds from the fruit were separated from the pulp, allowed to dry in air at room temperature (about 20 °C) and stored dry in brown paper envelopes at room temperature. About 400 of the resulting seeds were deposited with the NCIMB under reference 40015.

## EXAMPLE 2

Twelve seeds produced as in Example 1 were taken, grown to maturity and self-fertilized. Plant tissue from each of the 12 resulting seedlings was tested by Southern blot analysis as described in experiment 40 of European Patent Application No 87309853.7. Homozygotic and heterozygotic plants were identified. Seed from homozygous plants was collected, appropriately labelled and stored for future use. Seeds of one such plant, coded GR 105, was deposited with the NCIMB under reference 40134. Analysis of fruit of the plant GR 105 by the method of G A Tucker, N G Robertson, D Grierson, (European Journal of Biochemistry, 112 p 119-124, 1980) indicated an exceptionally low level of expression of the polygalacturonase enzyme, less than 1% of that in corresponding conventional tomato fruit at a similar stage of development.

Another way of obtaining homozygous seed would be to backcross GR16, or a similar transformant, or offspring thereof, to the untransformed Ailsa Craig variety. Standard genetic analysis then permits identification of parent seed homozygous for the antisense construct.

## Claims

1. Fruit having a reduced level of expression of one or more fruit-ripening enzymes, resulting from the presence in the plant genome of DNA capable of generating antisense RNA complementary to the RNA that generates such fruit-ripening enzymes. 5

2. Fruit as claimed in claim 1 that are strawberries, melons, cantaloups, peppers, or avocados.

3. Fruit as claimed in claim 3 wherein the fruit-ripening enzyme is selected from the following: xylanases;  $\alpha$ -galactosidase;  $\beta$ -galactosidase; cellulase ( $\beta$ -1,4-glucanase); peroxidase; hemicellulase; isoperoxidase; glucosidases; mannosidases; arabinosidases;  $\beta$ -fructofuranosidases; trehalase;  $\beta$ -xylosidase; acid phosphatase; rhaminase;  $\beta$ -1,3-glucanase; enzymes of calcium metabolism; arabinofuranosidase. 10 15

4. Fruit as claimed in claim 1 which are tomatoes and wherein the enzyme is a fruit-softening enzyme. 20

5. Tomatoes as claimed in claim 5 in which the fruit-softening enzyme is polygalacturonase or pectin methylesterase.

6. Seed of fruit claimed in any of claims 1 to 5. 25

7. Tomato seeds as claimed in claim 6 which are, or are descended from, the seeds deposited with NCIMB under the reference 40015.

8. A method of producing F1 hybrid tomatoes as claimed in claim 5 which comprises crossing two parent lines at least one of which is homozygous for a fruit-softening enzyme antisense construct. 30

9. A method as claimed in claim 8 in which one parent line is, or is descended from, the seeds deposited with NCIMB under the reference 40134. 35

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